Short Communication

An examination of an Antarctic soil metagenomic-derivate putative methylthioadenosine phosphorylase gene as a novel reporter gene for promoter trapping

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The reporter genes are widely used in promoter-trapping vectors for easy and rapid identification of the putative promoter sequences in cloned DNA fragments and in some cases to measure the promoter strength. In this aim, the reporter genes such as a *gfp* gene from *Aequorea victoria* encoding Green Fluorescent Protein (Miller et al., 2000; Pothier et al., 2007; Rasko et al., 2007), a *lacZ* gene encoding *E. coli* β -galactosidase (Fan et al., 2009; Li et al., 2007; Zhang et al., 2007), and a *luxAB* gene encoding *Vibrio fischeri* luciferase (Aoki et al., 2002; Kunert et al., 2000) were used. Moreover, the genes coding for antibiotic resistance were utilized in screening for the promoter sequences; e.g., a *cat* gene encoding chloramphenicol acetyltransferase was applied in the isolation of lactococcal (Jeong et al., 2006), mycobacterial (Das Gupta et al., 1993), cyanobacterial (Marraccini et al., 1993) and streptococcal (Kili et al., 1999) promoter sequences. Although the genes mentioned above are commonly used in many promoter-trapping vectors, scientists are still looking for new ones dedicated for searching out promoters in particular hosts. Cui et al. (2004) reported

the successful using of the methyl parathion hydrolase gene from *Plesiomonas* sp. strain M6 as a reporter gene to clone the promoter sequences, with different promoting strength, derived from the *Bacillus subtilis* 168 genomic DNA.

The *rsfp* gene was discovered during examination of an Antarctic soil-derived metagenome library and encodes a bacterial methylthioadenosine phosphorylase (GenBank accession number GQ202582). In a previous study, we found that *E. coli* colonies expressing the *rsfp* gene reveal strong pink fluorescence after exposure to UV light when grown on culture medium supplemented with rhodamine B (Cieřliński et al., 2009). In contrast to that, the colonies of the *E. coli* strains, deficient in the *rsfp* gene, do not show any fluorescence under the same experimental conditions. We found that the reason for the fluorescence phenomenon of *E. coli* cells expressing the *rsfp* gene is the binding of the fluorescent dye rhodamine B (abbrev. RB) molecules on RSFP protein molecules that leads to the increase in the fluorescence yield of RB molecules excited by UV light and the accumulation of RB molecules in *E. coli* cells during colony growth (manuscript in preparation).

Based on these results we decided to examine the potential of the *rsfp* gene as a novel reporter gene for identifying the location of the putative promoter sequences in the *E. coli* TOP10F' genome fragments

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cloned to a pTrap vector constructed in this work. The *E. coli* TOP10F' strain was used as model organism in this study. On the other hand, because of the expression of the *rsfp* gene in the *E. coli* cells leads to the accumulation of the rhodamine B that is the fluorescent dye, we also examined our new RSFP-RB reporter system by fluorescence microscopy.

In this aim, the *rsfp* gene was inserted into the *Bam*HI and *Sal*I restriction sites of the plasmid pUC19 in the opposite transcriptional direction to the *lacZ* promoter sequence on the plasmid. The primers F_19primer: 5'-TTAGGATCC**AGGAG**ATATACTTATGACTTCAAACG CAGTAGAA-3' (43 nt, *Bam*HI recognition site—underlined, RBS sequence—boldfaced) and R_primer: 5'-AAT GTCGACTACGGAAGTAATGCTGCAAGT-3' (30 nt, *Sal*I recognition site—underlined) were used to clone the Shine-Dalgarno sequence and the *rsfp* gene without the native promoter sequence from the plasmid pPINKuv (Cieśliński et al., 2009). The purified PCR products (A&A Biotechnology, Poland) were digested with *Bam*HI and *Sal*I (Fermentas, Lithuania) and cloned into the corresponding sites in pUC19 plasmid (Fermentas, Lithuania). The resulting plasmid was selected with the use of restriction analysis and the DNA insert was sequenced on both strands to ensure that no mutation had been introduced into the *rsfp* gene sequence during the course of PCR amplification. The resulting plasmid was named pTrap (Fig. 1).

Afterwards, *E. coli* TOP10F' genomic DNA fragments were cloned upstream of the promoterless *rsfp* gene of the pTrap vector. In this aim, the genomic DNA isolated from *E. coli* TOP10F' was digested with *Bsp*143I (*Sau*3AI) restriction enzyme (Fermentas, Lithuania) under optimal conditions for generating DNA fragments mainly in the 200–800 bp range. Next, the resulting DNA fragments were ligated to the *Bam*HI-digested pTrap vector. The ligation mixture was transformed into competent *E. coli* DH5 α and the cells were spread on LB agar containing 100 μ g ml⁻¹ of ampicillin, 0.1 mM IPTG and 0.001% of rhodamine B (Sigma-Aldrich, USA). Moreover, *E. coli* DH5 α /pUC19 strain and *E. coli* DH5 α /pUC19-RSFP strain bearing the *rsfp* gene under *Plac* promoter control (Cieśliński et al., 2009) were spread on the same agar medium as a negative and a positive control of the pink fluorescence of the recombinant clones, respectively. The Petri plates were incubated at 37°C for 18 h. Afterwards, the screening for the *E. coli* colonies exhibiting pink fluorescence as a result of *rsfp* gene expression was per-

Fig. 1. The physical map of the pTrap vector.

Abbreviations, rep(pMB1): *bla*, *rsfp* and *Plac* represent the sequence coding for pMB1 (ColE1) replication origin, ampicillin resistance gene, promoterless *rsfp* gene and *lacZ* promoter, respectively.

formed after exposure to UV light of 312 nm (Fig. 2). The screening revealed that 11 *E. coli* colonies out of 1,150 showed the pink fluorescence; then recombinant plasmids were isolated (A&A Biotechnology, Poland) and named pP1–pP11, respectively.

The genomic *E. coli* fragments trapped in pTrap derivative plasmids isolated from fluorescent clones were sequenced (Genomed, Poland) and the obtained DNA sequences were analyzed for the presence of DNA fragments of vector origin with VecScreen program and then aligned using BLAST software against the GenBank database and the achieved results confirmed that all analyzed DNA sequences contained different fragments of the *E. coli* genomic DNA with the sequence similarity of 99% to the well-defined DNA regions of *E. coli* DH1 genome sequence (GenBank accession no. CP001637.1). However, the further detailed analysis reveals that only the DNA inserts of pP2, pP5 and pP7 are single DNA fragments of *E. coli* genomic DNA, respectively. In the case of the DNA insert of pP1 plasmid we found out that this is a single DNA fragment of *E. coli* F plasmid DNA. Moreover, we found out that the DNA inserts of the other analyzed plasmids had been arising after the random ligation of two, four or even five different DNA fragments of *E. coli* ge-

Fig. 2. The recombinant *E. coli* DH5 α colonies exhibiting fluorescence under UV light (312 nm).

The positive fluorescent colonies appear pink.

genomic DNA with the sticky ends produced by *Sau*3AI enzyme (Table 1). Therefore, due to the possibility of the prediction of the artificial promoter sequence, each of the DNA fragments present in the DNA inserts of pP3, pP4, pP6, pP8–pP11 plasmids was analyzed separately for the presence of putative promoter regions.

First of all, the putative promoter sequences were predicted and analyzed by the BProm program optimized for identification of bacterial sigma70-dependent promoter sequences (major *E. coli* promoter class). BProm has about 80% accuracy in *E. coli* promoter recognition as was described at its website. The recent version of BProm is accessible from the website (<http://linux1.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb>). The BProm program revealed the presence of “promoter-like” regions in almost all DNA sequences under study (Table 1). Finally, these results were compared with Promoter Data Sets of RegulonDB version 7.4 that allowed us to make conclusions about the promoter trapping with the *rsfp* gene as a reporter. RegulonDB version 7.4 is based on RegulonDB version 7.0 (with later updates), described by Gama-Castro et al., 2011. However, in a few cases, this approach failed because of the lack of analyzed DNA sequence homology with the *E. coli* strain K-12 substrain MG1655 genomic DNA sequence

(NC_000913.2) used as a reference sequence for RegulonDB (Table 1). To summarize, 5 of 26 putative promoter sequences predicted with the BProm program correspond to the existing promoter sequences presented in the Promoter Data Sets of RegulonDB (Table 2). Moreover, we also found out that the sequence of putative promoter P1 corresponds to the sequence of the *PrepE* promoter of the *E. coli* F plasmid that was characterized by Wada et al., 1987 (Table 2).

Each of the other putative promoter regions was analyzed in terms of its location and orientation to the adjacent gene (or ORF) located on the same DNA strand of *E. coli* genomic DNA. We analyzed the location of the predicted promoter while keeping in mind that most promoters are located within 150 bp of the protein coding sequence (technical note to the BProm program). Moreover, we also compared the values of the linear discriminant function (LDF) parameters of the existing promoters presented in Table 2 with the LDF parameters of the other putative promoters presented in Table 1. The LDF parameter combines characteristics describing functional motifs and oligonucleotide composition of the potential transcription start positions of bacterial genes regulated by sigma70 promoters. A high value of an LDF parameter means a good fit to a linear combination of the features which characterize the existing sigma70 promoter regions. Table 3 shows a complete result of this analysis. The boldfaced lines in Table 3 indicate the predicted promoter regions. For example, the P3A promoter could be matched to an unidentified *fdoGHI-fdhE* operon promoter (RegulonDB). In conclusion, the presented results reveal the presence at least one highly probable promoter region in each analyzed DNA sequence that could control the expression of the *rsfp* gene as a reporter gene in the cells of recombinant *E. coli* strains.

On the other hand, we decided to verify the results of screening for the fluorescent colonies by the epifluorescence microscopy of the *E. coli* cells expressing the *rsfp* gene. In this aim, the cells of one of the resultant recombinant *E. coli* strains transformed with the p5 plasmid (randomly selected) and the pUC19 plasmid (negative control) were examined by fluorescence microscopy after being grown at 37°C for 18 h on LB agar plates supplemented with 100 $\mu\text{g ml}^{-1}$ of ampicillin and 0.001% of rhodamine B. In this aim, the colonies from 5 LB agar plates, each from *E. coli* DH5 α /p5 and from *E. coli* DH5 α /pUC19, were collected separately from the surface of the agar and resuspended

Table 1. The putative promoter sequences predicted by the BProm program (—35 sequences are boldfaced; —10 sequences are underlined) in the different DNA fragments of *E. coli* TOP10F genomic DNA cloned to the pTrap vector. LDF threshold for promoters = 0.20.

Plasmid name (DNA insert size)	Fragment name (DNA fragment size)	Reference DNA seq. (Data base and accession No.)	Position of the DNA insert/fragment in the reference DNA seq.	Query coverage %	Query identity %	Putative promoter name	Putative promoter region predicted with BProm program	LDF
pP1 (241 bp)	n/a	Plasmid F DNA, complete sequence (GenBank: AP001918.1)	48,196–48,436 bp	100	100	P1	TTGACTCTTTTATTATTAGTGTGACAAT CTAAAAAC	6.71
pP2 (243 bp)	n/a	<i>E. coli</i> str. K-12 substr. MG1655 (NCBI Reference Seq.: NC_000913.2)	3,188,647–3,188,405 bp	100	100	P2	CTGAAAAGTTGACGGAAACATTTTTAAA CTGCGTATT	5.82
pP3 (820 bp)	"A" (660 bp)	As above	4,084,472–4,083,813 bp	100	100	P3A	TTGACGAAACACCTGTGATATTGTGTCAC ATTTTCGATTT	4.26
	"B" (164 bp)	As above	3,596,646–3,596,483 bp	100	100	P3B	TTGGGTTGCCGCCAAATTGCTAATAATC GAGTACG	2.33
						P4A1	GTGACATGAACATAACAATCCGCGGG CAGATTCTTGACG	1.13
pP4 (1,232 bp)	"A" (1,004 bp)	<i>E. coli</i> str. K-12 substr. DH10B (GenBank: CP000948.1)	1,362,832–1,363,835 bp	99	100	P4A2	TTGACAGCGCGTTTGAACACGGAAAA GCTGTCGGCTG	0.73
						P4A3	TTGATAAAACACTATCAACGAGCCATA ATAAATCTGC	5.45
	"B" (231 bp)	<i>E. coli</i> str. K-12 substr. MG1655 (NCBI Reference Seq.: NC_000913.2)	2,218,332–2,218,562 bp	100	100	P4B1	TTGATTGCGGCGCTGAAAGCCACCG GTAACCGCTGGTGC	1.14
pP5 (1,643 bp)	n/a	As above	1,262,486–1,262,869 bp	100	100	P5	TTAATGCTAGCGTTACCGTCGGCTATC GTCTATGTT	3.23
	"A" (1,028 bp)	As above	934,356–935,387 bp	100	99	P6A1	CTGAACCTGGCTCGTCAGTTTGC GCAACAACA	0.57
						P6A2	TTGGATAACGCCCAATTCGCGGATAAT CCGTCGCC	2.15
pP6 (1,643 bp)	"B" (129 bp)	As above	630,166–630,215 bp	100	100	P6B	TCGCCACGAAGGATTCCATTAAACATC CCACCGT	0.57
	"C" (312 bp)	As above	1,530,734–1,530,986 bp	82	98	P6C	TTTTCAGGGATACGGCACATTGCTATT AATATTTTGACGA	3.45
	"D" (182 bp)	As above	1,659,822–1,660,003 bp	100	100	P6D	TTTACATGGACAGATGCCATCGACCA TGTACGGA	0.55

Plasmid name (DNA insert size)	Fragment name (DNA fragment size)	Reference DNA seq. (Data base and accession No.)	Position of the DNA insert/fragment in the reference DNA seq.	Query coverage %	Query identity %	Putative promoter name	Putative promoter region predicted with BProm program	LDF
pP7 (970 bp)	n/a	As above	375,863–376,832 bp	100	100	P7.1	<u>TACACCTACGGCGTTTACAGGTATACT</u> CGCTAAAA	3.16
						P7.2	<u>TTGCCAATGGCGATATTGGTTTTAACT</u> TCACTGACG	2.91
						P7.3	<u>TTCACGCCGCGCATCCGGCAGTCGTGTC</u> TATTATCAACGCAT	6.48
						P8A	<u>TCGAAAACAGTCACCCGAACCTTTTT</u> GTTATAGCTATCCGCA	1.22
pP8 (1,431 bp)	"A" (169 bp)	As above	1,132,280–1,132,448 bp	100	100	P8B	<u>TTGTTGTGTGACGAAAGCGGGAAC</u> TATCCGGCTCTG	0.62
	"B" (156 bp)		290,913–291,068 bp	100	100	P8C	<u>TTGTTTTCGCTATTTCAGCACCACCA</u> CAAAATGGCCTTCA	1.16
	"C" (124 bp)		2,153,008–2,153,131 bp	100	100	P8D	<u>TTTTCTTCCCGTTTCCGGTTTGTCI</u> AAAAATCCATGCTG	4.28
	"D" (315 bp)		1,361,456–1,361,770 bp	100	100	P8E1	<u>TTCGAACTGTAAGCCTGTATGGGCAT</u> CCTGATTTTGA	1.70
pP9 (351 bp)	"E" (683 bp)	<i>E. coli</i> str. K-12 substr. DH10B (GenBank: CP000948.1)	3,945,561–3,946,243 bp	100	100	P8E2	<u>TCTCCGCAAAATTTATTTGTCGTTATGA</u> TTTAAATGT	6.91
	"A" (253 bp)	As above	251,828–252,080 bp	100	100	P9A	<u>TGGAAGACAACTGGTGCTGGGATTA</u> TGATGTATACTA	2.54
	"B" (102 bp)		2,298,943–2,299,044 bp	100	100	–	Number of predicted promoters = 0	–
	"A" (81 bp)		3,514,801–3,514,881 bp	100	100	–	As above	–
pP10 (278 bp)	"B" (201 bp)		621,454–621,654 bp	100	100	P10B	<u>TTGCCATCAAATATTTTAAATACCTATT</u> CTTAGGCTGC	4.46
	"A" (254 bp)	As above	328,532–328,785 bp	100	100	P11A	<u>TTATTGATTGGACGTTCAATATAAAATG</u> TGCTTTA	3.02
	"B" (90 bp)		3,355,641–3,355,730 bp	100	100	–	Number of predicted promoters = 0	–

Table 2. The existing promoters revealed during the analysis of the DNA sequences of the selected inserts/fragments presented in Table 1 with the BProm program and promoter data sets of RegulonRB.

Plasmid name	Number of predicted promoter regions in the analyzed plasmid DNA insert	Putative promoter name [BProm]	LDF [BProm]	Existing promoter name	Reference DNA seq. (Data base and accession No.)	Position +1 bp	Reference
pP1	1	P1	6.71	repE	Plasmid F DNA, complete sequence (GenBank: AP001918.1)	48,425	Wada et al., 1987
pP5	1	P5	3.23	hemAp2	<i>E. coli</i> str. K-12 substr. MG1655 chromosome (NCBI Reference Seq.: NC_000913.2)	1,262,806	Choi et al., 1996 Verkamp and Chelm, 1989
pP6	4	P6C	3.45	pptAp7	As above	1,530,882	Huerta and Collado-Vides, 2003
pP9	1	P9A	2.54	yafNp	As above	251,969	Christensen-Dalsgaard et al., 2010 Zaslaver et al., 2006
pP10	1	P10B	4.46	fepDp1	As above	621,457	Shea and McIntosh, 1991
pP11	1	P11A	3.02	betTp	As above	328,639	Lamark et al., 1996

by vortexing in 1 ml of phosphate buffered saline (PBS) pH 8.0. The cells resuspended in PBS were centrifuged ($4,000 \times g$, 6 min, room temperature) and then the supernatant was removed. Next, the pellets of the cells were washed with 1 ml of PBS and centrifuged (as before) after vortexing. This procedure of washing cells with PBS was carried out three times in order to get rid of the remaining LB agar. After the last step of washing, the cells were resuspended in 1 ml of PBS and this solution was subjected to 10-times serial dilutions in PBS buffer. A 25 μ l aliquot of diluted homogenate solutions of 10^{-2} , 10^{-3} , 10^{-4} and 10^{-5} of initial concentration was placed on the microscope slides and allowed to dry. After drying, the slides were covered with a cover slip and sealed with nail polish to avoid desiccation of specimens as well as to prevent them from any negative influence of environmental conditions. The specimens were observed under the green light (wavelength of 540 nm) on the fluorescent microscope Olympus BX60, magnification x60. In contrast to *E. coli* cells harboring the pUC19 plasmid that did not reveal any red fluorescence (Fig. 3A), the cells harboring p5 plasmid (Fig. 3B) showed strong red fluorescence.

In conclusion, the presented results confirm that the *rsfp* gene can be useful as a new reporter gene for the screening of putative promoter sequences in bacterial hosts. Moreover, we confirmed the localization of RSFP protein and the accumulation of RB molecules in the bacterial cells of the *rsfp* gene expressing *E. coli* strains constructed during this work after growth on LB agar plates supplemented with RB. The rhodamine B fluorescent dye and the metagenomic-derivate RSFP protein is a new reporter system useful for promoter trapping studies, as well as at fluorescence microscopy. Therefore, in future we are going to use the analogous approach and the *rsfp* gene for studying the genetics of cold-adapted bacteria that have been in the limelight for our scientific team for the last decade. In our opinion, the advantage of the presented reporter system in comparison to other reported systems based on using such above-mentioned report genes as genes as *lacZ* or *cat* for bacterial promoter trapping is that this system allows for simple observation of *rsfp* gene expression in living bacterial cells by using the fluorescent dye rhodamine B and the epifluorescence microscopy method. On the other hand, in contrast to the reporter systems based on the use of the fluorescent proteins such as GFP protein, the necessity of the

Table 3. The highly probable promoters (boldface fonts) and the putative promoters with negative prediction results (normal fonts) revealed during the analysis of the DNA sequences of the selected inserts/fragments presented in Table 1 with BProm program and promoter data sets of RegulonRB.

Plasmid name	Putative promoter name (BProm)	LDF	Putative promoter is located between two neighboring ORFs/genes	Putative promoter is placed in forward orientation with adjacent gene/ORF located on the same strand	Adjacent ORF name (forward orientation)	The distance between promoter and adjacent gene/ORF (forward orientation)	Putative transcription unit
pP2	P2	5.82	No (overlapping with yqjH gene)	Yes	yqjI	117 bp	yqjI
	P3A	4.26	Yes	Yes	fdoG	223 bp	fdoGHI-fdhE
pP3	P3B	2.33	No (partially overlapping with <i>livJ</i> gene and a repeat region: RIP259)	No	n/a	n/a	n/a
	p4A1	1.13	Yes	Yes	ecdH10B_1333	35 bp	ecdH10B_1333
	p4A2	0.73	No (overlapping with ECDH10B_1333 ORF)	Yes	ecdH10B_1334	562 bp	ecdH10B_1334
p4	p4A3	5.45	Yes	Yes	ecdH10B_1334	102 bp	ecdH10B_1334
	p4B1	1.14	No (overlapping with <i>bgIX</i> gene)	Yes	<i>osmF</i>	1,826 bp	n/a
	p6A1	0.57	No (overlapping with <i>ftsK</i> gene)	Yes	<i>loiA</i>	2,070 bp	n/a
p6	p6A2	2.15	No (overlapping with <i>ftsK</i> gene)	Yes	<i>loiA</i>	801 bp	n/a
	p6B	0.57	No (overlapping with <i>cstA</i> gene)	No	n/a	n/a	n/a
	p7.1	3.16	Yes	Yes	yail	34 bp	yail
p7	p7.2	2.91	No (overlapping with <i>yail</i> gene)	No	n/a	n/a	n/a
	p7.3	6.48	Yes (partially overlapping with a REP32 sequence)	No	n/a	n/a	n/a
	P8A	1.22	No (overlapping with <i>flgE</i> gene)	No	n/a	n/a	n/a
	P8B	0.62	No (overlapping with <i>yagJ</i> gene)	No	n/a	n/a	n/a
	P8C	1.16	No (overlapping with <i>mdtA</i> gene)	No	n/a	n/a	n/a
p8	P8D	4.28	No (overlapping with ORF signed 15)	No	n/a	n/a	n/a
	P8E1	1.70	No (overlapping with <i>hdfR</i> gene)	Yes	<i>yifE</i>	461 bp	<i>yifE</i>
	P8E2	6.91	Yes	Yes	yifE	85 bp	yifE

Fig. 3. The comparison of the fluorescence microscopy results for *E. coli*/pUC19 cells (dilution 10^{-2} ; 3A) and *E. coli*/p5 cells (dilution 10^{-2} ; 3B).

use of fluorescent dye in our system seems to be a major disadvantage of the proposed solution. However, despite this disadvantage we look forward to the possibility of developing of our reporter system. Rhodamine B is only one of the many purchasable fluorescent xanthene derivatives such as rhodamine 123, rhodamine 6G, eosin Y, eosin B or fluorescein commonly used in science and industry. What is especially important for us is that each of these dyes has different spectroscopic excitation and emission spectra. Therefore, in our next study we will examine them as alternative fluorescent partners for RSFP protein that could expand the usefulness of RSFP protein as a reporter in living bacterial cells observed with the epifluorescence microscopy method.

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